TOXICOLOGY AND APPLIED PHARMACOLOGY 135, 67–76 (1995)

Effects of Developmental Hypothyroidism on Auditory and Motor Function in the Rat[1]

ELLEN S. GOLDEY,*[,2] LAURA S. KEHN,* GEORGIA L. REHNBERG,† AND KEVIN M. CROFTON*[,3]

*Neurotoxicology Division and †Developmental Toxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Received February 6, 1995; accepted June 25, 1995

Effects of Developmental Hypothyroidism on Auditory and Motor Function in the Rat. GOLDEY, E. S., KEHN, L. S., REHNBERG, G. L., AND CROFTON, K. M. (1995). *Toxicol. Appl. Pharmacol.* 135, 67–76.

Deafness is a common result of severe hypothyroidism during development in humans and laboratory animals; however, little is known regarding the sensitivity of the auditory system to more moderate changes in thyroid hormone homeostasis. The current investigation compared the relative sensitivity of auditory function, motor function, and growth to the effects of moderate to severe perinatal hypothyroidism in the rat. Rats received propylthiouracil (PTU) in drinking water at concentrations of 0, 1, 5, and 25 ppm from Gestation Day 18 until postnatal day (PND) 21, and the effects on their offspring were evaluated. At 1 ppm, PTU did not affect any of the measured endpoints. Serum thyroxin concentrations were sharply reduced in the 5 and 25 ppm PTU groups at all ages sampled (PND 1, 7, 14, and 21). Marked reductions in serum triiodothyronine (T3) concentrations were also detected for all ages ≥7 at 25 ppm PTU, whereas no effects of 5 ppm PTU on serum T3 were apparent until PND 21. Compared to the controls, pups exposed to the highest dose of PTU demonstrated a delay in eye opening, reduced body weights, decreased and/or delayed preweaning motor activity, and persistent, postweaning hyperactivity. Only slight and transient effects on eye opening and ontogeny of motor activity were seen at the intermediate dose of PTU (5 ppm). Reflex modification audiometry revealed that, compared to controls, adult offspring from the 5 and 25 ppm treatment groups showed dose-dependent auditory threshold deficits (35 to >50 dB) at all frequencies tested (1, 4, 16, 32, and 40 kHz). Such dose-dependent effects indicate that the developing auditory system may be sensitive to mild hypothyroidism, suggesting the possible need for routine audiometric screening for infants and children at risk for iodine deficiency, myxedema, and/or exposure to thyrotoxic environmental agents.

Congenital hypothyroidism is a relatively common disorder in the human population, with estimates in developed countries ranging from 1 in 3000 to 1 in 3800 live births (LaFranchi, 1993; Sorcini *et al.*, 1993). Deafness and mental retardation are characteristic of severely affected individuals (Boyages and Halpern, 1993), and it has been proposed that hypothyroidism occurring during the second trimester of fetal development is primarily responsible for the auditory, neurological, and intellectual deficits, whereas hypothyroidism occurring during the postnatal period causes the dwarfism and sexual immaturity commonly seen in these individuals (Delong, 1993; Boyages and Halpern, 1993; Boyages, 1993).

Rodents have been widely used as animal models for studying the effects of developmental hypothyroidism (see Dussault and Ruel, 1987 for review). The brain and auditory system of rats and mice undergo substantial development postnatally; therefore, induction of hypothyroidism during the perinatal period (late gestation until weaning) encompasses the homologous, thyroid-sensitive, stages of nervous system and auditory system development in humans (i.e., second trimester until birth). In rodents, marked reduction in thyroid hormones during perinatal development results in a number of effects on the brain including reduced brain size (Balázs *et al.*, 1968; Behnam-Rassoli *et al.*, 1991), abnormal myelination (Rodriguez-Péna *et al.*, 1993), arborization and synapse formation of neurons (Poddar and Sarkar, 1993), and neurotransmitter disruption (Vaccari *et al.*, 1990). Delays in eye opening (Comer and Norton, 1982), reflex development (Comer and Norton, 1982), and weaning (Blake and Henning, 1985) have also been reported, along with poor motor coordination (Narayanan *et al.*, 1982) and structural and functional auditory abnormalities (Deol, 1973; Uziel *et al.*, 1980, 1981, 1983, 1985). Whereas rodent offspring may recover from many of the neurological and somatic delays caused by developmental hypothyroidism, the effects on auditory structures appear to be profound and permanent (Uziel, 1986). Deafness is frequent in humans suffering the effects of severe developmental hypothyroidism (Fraser, 1965; Vanderschueren-Lodeweyckx *et al.*, 1983; Boyages and Halpern, 1993), but little is known regarding the relative

[1] Portions of the data contained herein were presented at the 1994 Neurobehavioral Teratology Society Conference. The manuscript has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

[2] Current address: Department of Biology, Wofford College, Spartanburg, SC 29303-3663.

[3] To whom correspondence should be addressed.

0041-008X/95 $12.00

sensitivity and persistence of effects on the auditory system compared to effects on other neurological targets.

Congenital hypothyroidism may result from a number of factors including dietary iodine deficiency (endemic cretinism), myxedema, and/or exposure to thyrotoxic environmental agents, and the degree of hormonal suppression is likely to vary accordingly. Infants and children may suffer a range of effects attributable to moderate to severe hypothyroidism; therefore, animal models which can detect thyroid hormone-related perturbations may have important clinical relevance. In the present investigation, the goitrogen, propylthiouracil (PTU), was used to compare the relative sensitivity of effects of perinatal hypothyroidism on auditory function, motor activity, and physical development.

METHODS

Animals

Primiparous Long–Evans rats arrived from Charles River (Raleigh, NC) on Gestation Day (GD) 13; the breeding date was GD 0. Animals were housed in an AAALAC-approved animal facility, and all experiments were approved in advance by the Health Effects Research Laboratory animal care committee of the U.S. Environmental Protection Agency. Pregnant animals were housed individually in standard plastic hanging cages (45 × 24 × 20 cm) with sterilized pine shavings as bedding. Animal rooms were maintained on a 12:12 hr photoperiod, L:D (0600:1800), and food (Purina Lab Chow) and water were provided *ad libitum*.

Beginning on GD 22, animals were checked twice daily (AM and PM) for births, and the date that birth was first discovered was assigned Postnatal Day (PND) 0. On PND 2, 2 male and 2 female pups, chosen randomly within a litter, were given foot tattoos for individual identification (Avery and Spyker, 1977) and returned to their natural dam. Only tattooed pups were used in subsequent behavioral evaluations. Nontattooed pups within a treatment group were pooled on PND 2 and randomly fostered to dams within the same treatment so that all litters contained 10 pups. Only nontattooed pups were selected for subsequent tissue collection, and litter size was correspondingly reduced by 1 pup at each collection age. Because weaning is delayed in pups receiving high dosages of PTU (Blake and Henning, 1985), dams were not removed from their litters until PND 26, at which time tattooed littermates were housed in gender-matched pairs.

Dosing

6-*n*-Propyl-2-thiouracil (Sigma Chemical, St. Louis, MO; 99.3% purity) was administered to the dams via drinking water. Drinking water solutions were mixed vigorously for at least 24 hr prior to use to ensure that all of the PTU had dissolved. PTU exposure was initiated on GD 18 and continued until PND 21. Pregnant rats were assigned to one of four treatment groups (10 rats per group): 0, 1, 5, or 25 ppm PTU, respectively. Each bottle was weighed prior to, and after, each addition of drinking water solution which occurred twice per week. An estimate of daily water consumption was calculated from the amount (ml) of water consumed over the 3- or 4-day period. Subsequently, an estimate of daily dose (mg PTU/kg body wt) was calculated using the following formula: [average daily water consumption (ml)·PTU concentration (mg/ml)] ÷ total biomass in cage (kg). Daily dosages were estimated on a total biomass (dam weight + litter weight) basis for two reasons: (1) dams with larger pups and/or litters will drink more water than those with smaller and/or fewer pups, and (2) pups may drink directly from the bottles during the latter part of the preweaning period.

Neonatal Growth and Development

Pups were weighed (by litter) on PND 1, twice per week until PND 26 (weaning), and weekly thereafter. Maternal body weights were recorded twice per week throughout the dosing period. Eye opening was monitored daily beginning on PND 13. The number of pups within a litter with at least one eye open was recorded each day until all pups' eyes were open. Observations of overtly abnormal physical appearance and/or behavior were noted throughout the study.

Thyroxin (T4) and Triiodothyronine (T3) Determination

One or two nontattooed pups per litter were decapitated on PND 1, 7, 14, and 21, and blood was collected in microcentrifuge tubes. The tubes were immediately placed on ice, the blood was allowed to clot for up to 2 hr, and the tubes were then centrifuged at 2500 rpm for 15 min. Serum was transfered to microfuge tubes and stored at −80°C until analysis of total T3 and T4 by radioimmunoassay (kits from Diagnostic Products, Inc., Los Angeles, CA). If more than one pup per litter was sampled, a mean value for the litter was calculated and used in subsequent statistical analyses. The inter- and intraassay coefficients of variation were below 10% for both hormone assays.

Figure-Eight Maze Activity

One tattooed male and female pup per litter were tested individually in one of 16 automated figure-eight mazes for 30 min on PND 13, 15, 17, 19, and 21, and for 1 hr on PND 30, 40, 50, 75, and 120 (repeated testing of the same pups). Motor activity was detected by eight infrared photobeam pairs which transected a series of interconnected alleys (10 × 10 cm) converging on a central arena (in a figure-eight pattern) and covered by an acrylic plastic top (Ruppert *et al.*, 1984a). Within a test day, time of testing, balanced across treatment groups, occurred between 0900 and 1600 hr. Data were analyzed in two ways. First, data were summated in 5-min intervals for analysis of within-session habituation (i.e., decrease in activity within the test session). Data were also analyzed as total counts per test session and compared across test ages. A separate repeated measures ANOVA was performed on total counts/session across each of the two age blocks (i.e., across 30-min sessions for pups ≤PND 21 and across 1-hr sessions for animals ≥PND 30).

Acoustic Startle Response (ASR)

Habituation of ASR in preweanlings. Habituation to the ASR was assessed in one tattooed male and female pup per litter on PND 24. Testing was conducted in eight sound-attenuated chambers, each containing a wire-mesh, plastic-bottom test cage, mounted on a load cell/force transducer assembly designed to measure vertical force (Ruppert *et al.*, 1984b). Each rat was placed in a test cage and, following a 10-min acclimation period at an ambient noise level of 30 dB (A weighted), received a total of 50 trials (intertrial interval = 15 sec). The startle eliciting stimulus (S2) was a 120-dB, 40-msec white noise burst (2.5-msec rise/fall). Data collection began with the presentation of the eliciting stimulus and continued for 64 msec. The analog signal for each response (startle movement) was digitized at 1 kHz and converted to grams using a previously determined calibration curve for each load cell. Response amplitude, taken from each animal's average response curve, was calculated across trials within a block. Ten sequential blocks of 5 trials were used to assess habituation (decreased response amplitude across trials) to the eliciting stimulus. The mean response amplitude across all trial blocks was also calculated for treatment comparisons.

Reflex modification audiometry in adult offspring. Auditory thresholds were determined beginning on PND 75 utilizing reflex modification audiometry (Young and Fechter, 1983; as modified by Crofton *et al.*, 1990). Testing was conducted within the same apparatus described previously for the acoustic startle habituation. Eight tattooed male rats and eight tattooed

female rats (one per litter per sex) were evaluated from each exposure group. Each rat was placed in a test cage and, following a 10-min acclimation period at an ambient noise level of 30 dB (A weighted), received a total of 240 trials. Each trial consisted of an invariable eliciting stimulus (S2; 120-dB SPL, 40-msec burst of broadband white noise with a 2.5-msec rise/fall), preceded by a prepulse tone (S1) at one of five frequencies (1, 4, 16, 32, or 40 kHz). The trials were arranged in 10 blocks, with 24 trials per block. Each block contained a blank control trial, during which only S2 was presented, and 23 trials in which a different intensity (−6 to 90 dB SPL, in increments of 3 or 6 dB) of the S1 stimulus (40-msec duration, 2.5-msec rise/fall) was presented 90 msec prior to the S2 stimulus. The order of presentation of the trials was computer generated in a semirandom, but balanced, fashion so that within each of 10 24-trial blocks, each S1/S2 condition was presented once. The intertrial interval was 15 sec. Data collection and analog signal processing were similar to those described previously for ASR habituation testing. Auditory thresholds were estimated using a nonlinear regression procedure (SAS, 1989). Auditory thresholds were defined as the S1 intensity above which the response to S2 was inhibited (see Crofton, 1992). Animals were tested for one S1 frequency per day, with the five S1 frequencies randomly assigned across test days. The startle response amplitude was also calculated as the mean response amplitude across all blank control trials.

Acoustical measurements were made with a Bruel and Kjaer (B&K) 2610 measuring amplifier equipped with a B&K 4135 0.25-in. microphone, or a B&K 2209 Impulse Level Meter equipped with a B&K 4165 0.5-in. microphone (dB re: 20 μPa). Ambient noise (20 sec averages, with a 22.4-Hz high-pass filter) in the animal colony averaged 78 dB overall SPL with most power at the low frequency end (<0.2 kHz), and a 40-dB drop from 0.2 to 10 kHz. Test chamber background noise averaged 28.4 dB (A weighted), again with most power at the low frequencies (see Crofton and Zhao, 1993).

Data Analysis

Analysis of variance (ANOVA) procedures were used for all main and simple-effects tests (SAS, 1989). In the case of more than one independent variable, significant interactions were followed by simple-effects ANOVA tests for each independent variable. Repeated measures ANOVAs (multivariate) were used where appropriate [i.e., to compare body weights and activity counts (across age), acoustic startle habituation (across trial block), and auditory thresholds and startle amplitudes (across the five S1 frequencies)]. When a male and female pup from the same litter were tested, gender was a within-litter repeated measure (to control for possible "litter effects"), whereas treatment was a between-litter factor for all variables. For each significant effect of treatment, mean contrast comparisons were made using Duncan's new multiple range test. An alpha value of 0.05 was used for all comparisons.

RESULTS

Maternal Body Weight, Water Consumption, and Pup Development

There were no treatment-related differences in dam body weights throughout the dosing period. Water consumption was significantly decreased (28–36% below controls) only in the 25 ppm treatment group after PND 9 [$F(3,25) > 7$; $p < 0.05$; average (±SE) control consumption = 77 ± 8 ml/day from PND 9 to PND 21]. Estimates (mean ± SE) of daily dose were 0.17 ± 0.012 and 0.86 ± 0.041 mg/kg in the 1 and 5 ppm groups, respectively. Estimates in the 25 ppm group decreased over time, from a high of 4.69 ± 0.25 mg/kg/day prior to parturition, to a low of 2.58 mg/kg/day

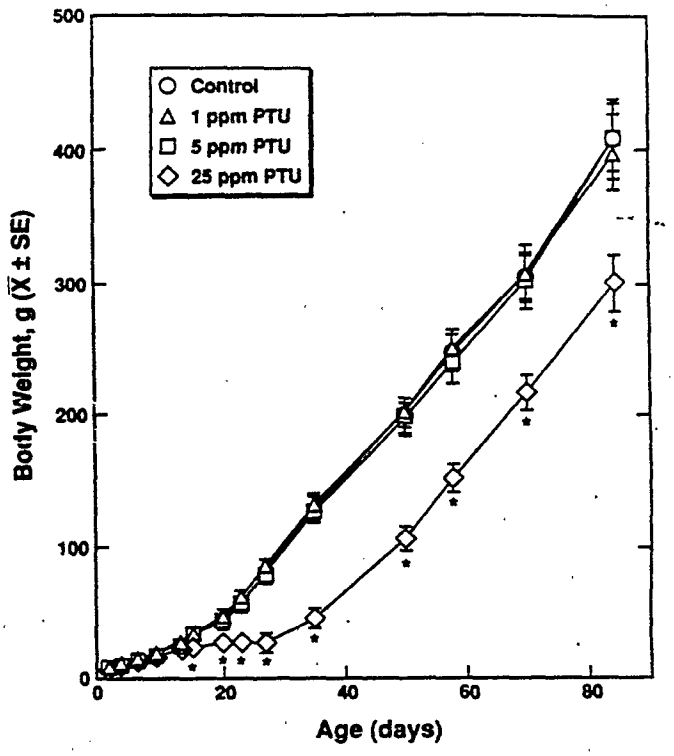

FIG. 1. Body weights (mean ± SE) of offspring exposed to 25 ppm PTU were significantly reduced compared to controls by PND 14, whereas the other treatments did not affect body weight gain. N = 6, 7, 8, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively. *Significantly different from the control value at the same age ($p < 0.05$).

on PND 21. Deficits in pup body weight became apparent in the 25 ppm treatment group during the second postnatal week, and these deficits persisted into adulthood (Fig. 1). The body weight findings were supported by a significant age by treatment interaction [$F(24,50) = 3.8$, $p < 0.05$], and significant effects of treatment at each age ≥PND 13 [all $F(3,27) \geq 8.4$; $p < 0.05$]. Mean contrasts revealed that offspring body weights were reduced in the 25 ppm group compared to controls on these days ($p < 0.05$). Body weights were not affected by the 1 or 5 ppm PTU treatments at any age.

Eye opening was delayed in PTU-treated animals in a dose-dependent fashion (Fig. 2). Whereas virtually all control and 1 ppm pups' eyes were open by PND 16, none of the pups' eyes from the 25 ppm group were open at this age, and it was not until PND 20 that all pups eyes from the 25 ppm group were open. The 5 ppm treatment group also showed a moderate delay in the age of eye opening compared to controls. These findings were supported by a significant interaction between age and dose [$F(15,120) = 12.5$; $p < 0.0001$], and significant treatment effects at each age from PND 15 to PND 19 [$F(3,27) \geq 3.3$; $p < 0.05$]. Mean contrast comparisons at each age indicated that eye opening for pups from the highest dose was significantly

delayed compared to controls on all days from PND 15 until all eyes were open on PND 20.

Although an effort was made to conduct observational assessments "blind" to treatment group, animals from the highest dose of PTU could be easily discerned from the other treatment groups. Not only were these animals notably smaller, but they had poor balance and coordination; they were most easily noted during the late preweaning period (e.g., pups which attempted to rear on their haunches would often topple over from their rearing stance). In addition, high-dose pups were observed to frequently hop about, particularly when physically disturbed such as upon opening the cage. One litter from the 25 ppm group did not survive after their dam was removed at weaning (PND 26), presumably due to their underdeveloped state. No observational differences were readily discernable at the lower (≤5 ppm) PTU doses.

T4 and T3 Determination

At all ages sampled, serum T4 concentrations were at or below the level of detection (≤1 ng/ml) in samples collected from animals in the 25 ppm PTU treatment group (Fig. 3, left). T4 concentrations were also significantly reduced in samples collected from the 5 ppm treatment group at all ages sampled. Lowered serum T3 concentration was detected in the 25 ppm PTU group at all sampled ages ≥PND 7, whereas T3 concentration was slightly reduced in the 5 ppm PTU group only on PND 21 (Fig. 3, right). For T4, these results were supported by a significant treatment by age interaction [$F(12,95) = 36$; $p < 0.05$], a main effect of treatment [$F(3,12) = 83$; $p < 0.05$] and of age [$F(3,12) = 107$; $p < 0.05$]. For T3, a significant treatment by age interaction [$F(12,93) = 65$, $p < 0.05$], a significant main effect of treatment [$F(3,12) = 59$; $p < 0.05$], and an effect of age [$F(3,12) = 294$; $p < 0.05$] were found. Step-down comparisons indicated a significant effect at each age sampled for T4 [$F(3,16-30) \geq 12$, $p < 0.05$], and for PND 7–21 for T3 [$F(16-26) \geq 10$, $p < 0.05$]. Results of mean contrast comparisons between the controls and PTU groups at each test age are indicated in Fig. 3 ($p < 0.05$). The lowest dose of PTU (1 ppm) did not affect the serum concentrations of either thyroid hormone at any age sampled.

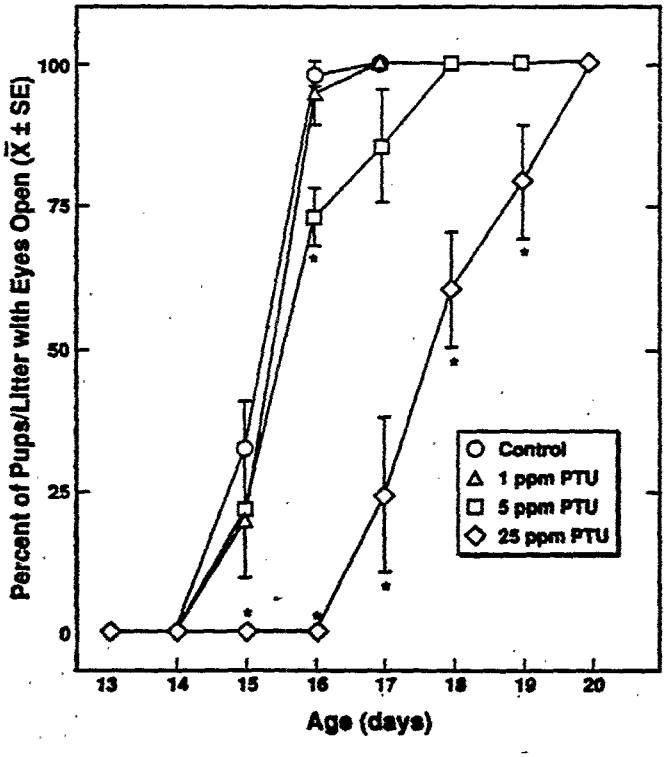

FIG. 2. Age of eye opening was determined as the percentage of all pups within a litter ($N = 6, 7, 8$, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively) with at least one eye open at each age (mean percentage ± SE). *Significantly different from the control value at the same age ($p < 0.05$).

Figure-Eight Maze Activity

PTU altered the ontogenetic profile of motor activity, with PTU-treated preweanlings demonstrating a dose-dependent decrease and/or developmental delay in motor activity at both the intermediate and highest PTU doses (Fig. 4, left). Pups exposed to 5 ppm PTU showed only early activity deficits at 15 days of age, whereas the highest PTU dose (25 ppm) caused reduced activity from PND 15 to PND 19. These findings were supported by a significant interaction between age and treatment [$F(12,66) = 6.59$; $p < 0.05$], significant effects of treatment on Days 15–19 [all $F(3,31) \geq 5.47$; $p < 0.05$], and significant means contrasts as indicated by asterisks in Fig. 4. There were no gender-dependent differences in performance in the figure-eight maze in preweanling animals and, as expected, activity significantly increased with age [$F(4,25) = 88.35$; $p < 0.05$]. In addition, from PND 17 through PND 21, high-dose pups showed a lack of habituation on each test day, rather than the within-session decreasing pattern shown by the other treatment groups (Fig. 5).

Treated animals demonstrated a distinctly different pattern of activity after weaning (Fig. 4, right). By PND 30, activity of animals exposed to 25 ppm PTU was elevated compared to the controls; an effect which was significant by PND 50 and persisted through the final test day (PND 120). These findings were supported by a significant treatment effect [$F(3,27) = 4.58$; $p < 0.05$], significant step-down ANOVAs at ages ≥PND 50 [all $F(3,30) \geq 3.37$; $p < 0.05$], and significant means contrasts as indicated in Fig. 4 ($p < 0.05$). There was no interaction between age and treatment, indicating that the treatment groups behaved similarly across all ages of testing. No effects on postweaning motor activity were detected for the 1 or 5 ppm PTU groups. There was a

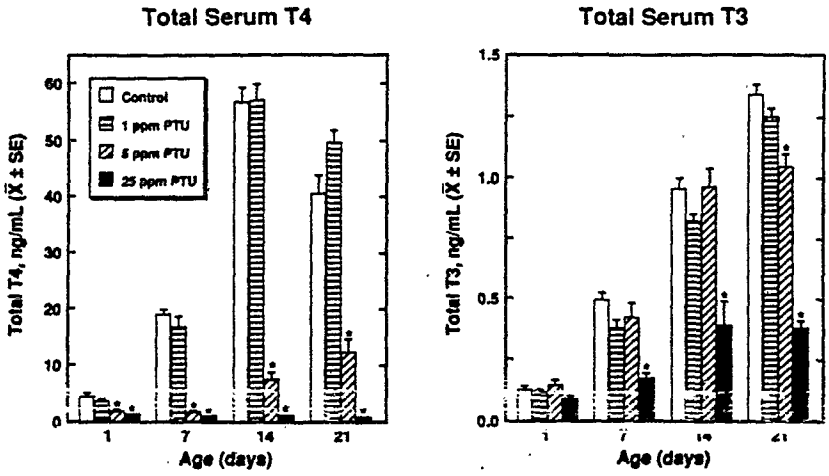

FIG. 3. PTU at 5 or 25 ppm dramatically lowered total serum T4 concentration at all ages sampled (left). T3 was significantly reduced by 25 ppm of PTU at all ages ≥7, whereas 5 ppm PTU caused a moderate reduction in T3 only on PND 21 (right). *Significantly different from the control value at the same age ($p < 0.05$).

significant effect of gender [$F(1,27) = 6.32$; $p < 0.05$] at the postweaning ages, with females being slightly more active than males. However, no significant interactions with gender were seen; therefore (as in other figures), results are graphically depicted based on "per litter" mean values. Within each test day, a similar pattern of habituation (decreasing activity across the 1-hr session) was shown by all treatment groups (data not shown).

ASR

Habituation of ASR in preweanlings. Interestingly, offspring exposed to 25 ppm PTU showed a complete absence of startle response at 24 days of age (Fig. 6). Animals exposed to 5 ppm PTU showed lower startle response amplitudes during the early trial blocks of acoustic startle testing, and their response did not appear to habituate across trial blocks. These results are supported by a significant interaction between trial block and treatment [$F(27,59) = 2.36$; $p < 0.05$], effects of treatment at each trial block [all $F(3,31) \geq 28.57$; $p < 0.05$], and subsequent means contrast comparisons for each treatment block. There were no gender-dependent effects on startle responsiveness or habituation. Startle amplitudes decreased (habituated) over the 50-trial session for the control and 1 ppm PTU groups; a finding supported

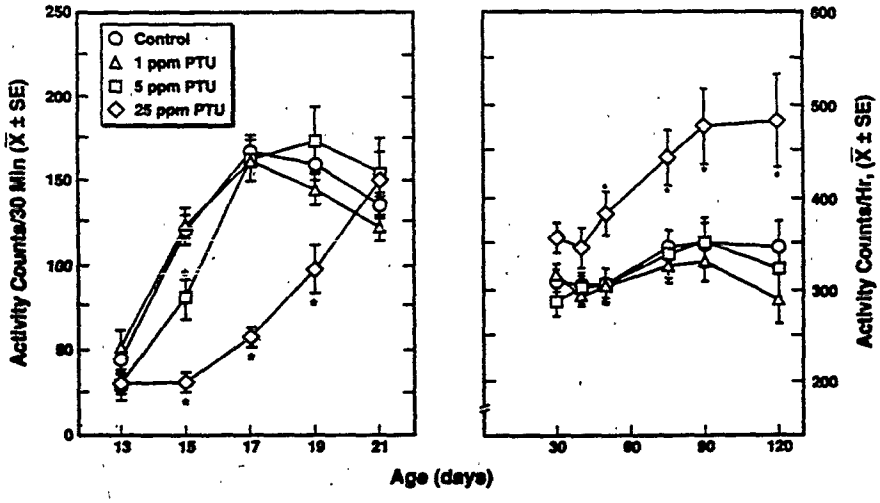

FIG. 4. PTU exposure caused reductions and/or developmental delays in figure-eight maze activity (mean ± SE) in preweanling animals (left). This effect was most apparent at the highest dose where activity was reduced compared to controls until PND 21. At the intermediate PTU dose, activity was reduced only on PND 15 compared to controls. Postweaning (right) animals exposed to PTU at the highest dose showed persistently higher activity counts/1 hr session in the figure-eight maze compared to the controls. $N = 6$, 7, 8, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively (one male and one female/litter). *Significantly different from the control value at the same age ($p < 0.05$).

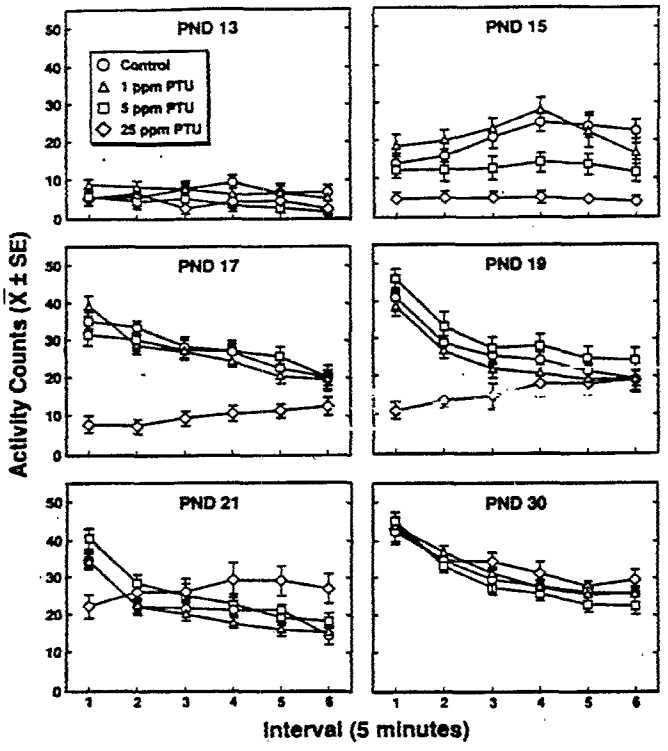

FIG. 5. Daily patterns of motor activity are shown for each day of preweaning figure-eight maze testing. The typical pattern of habituation to the test apparatus was demonstrated by pups from the controls and 1 and 5 ppm treatment groups by PND 17. However, pups from the 25 ppm PTU group did not habituate and appeared to be more active during the latter part of the 30-min test session. The first 30 min of testing on PND 30 is included to show that the pattern of habituation is more like that of controls at this age.

by a significant main effect of trial block [$F(9,20) = 5.11$; $p < 0.05$].

Acoustic startle response amplitude. Startle response amplitudes, sharply reduced on PND 24 in pups from the 25 ppm group (Fig. 7, left), were significantly elevated in adult animals from the 5 and 25 ppm PTU groups compared to the controls (Fig. 7, right). This finding in adults was supported by a significant effect of treatment [$F(3,27) = 8.70$; $p < 0.05$] followed by means contrast comparisons. Regardless of treatment, repeated measures analysis (across all days of testing) indicated that adult female startle amplitudes (e.g., control females = 72.1 g ± 17.3 SE) were significantly lower [$F(1,27) = 6.08$; $p < 0.05$] than those of males (e.g., control males = 123 g ± 44.8).

Reflex modification audiometry in adult offspring. Severe auditory deficits were apparent in animals exposed to 25 ppm PTU at all frequencies tested, and the intermediate dose of PTU (5 ppm) also produced significant, although more moderate, threshold shifts at all frequencies (Fig. 8). No effects on auditory thresholds were seen at the lowest dose of PTU (1 ppm). These effects were supported by a significant overall effect of treatment [$F(3,27) = 78.7$; $p < 0.05$] and significant effects of treatment at each frequency [all $F(3,30) \geq 28.15$; $p < 0.05$]. There was no interaction between frequency and treatment. No effect of gender, nor interactions with gender, was seen for auditory thresholds. The auditory threshold for a midfrequency tone (16 kHz) was lower than thresholds for higher and lower frequency tones, a finding supported by a significant main effect of frequency [$F(4,24) = 5.73$; $p < 0.05$].

DISCUSSION

The current investigation evaluated the dose–response characteristics of drug-induced perinatal hypothyroidism to compare the sensitivity of effects on physical development, motor activity, and auditory function. Whereas all of the measured endpoints were dramatically affected at 25 ppm PTU (a dosage which caused severe decreases in T4 and T3 during the preweaning period), the effects of 5 ppm PTU were more subtle. At this intermediate dose, pups showed only mild and transient effects on physical development and

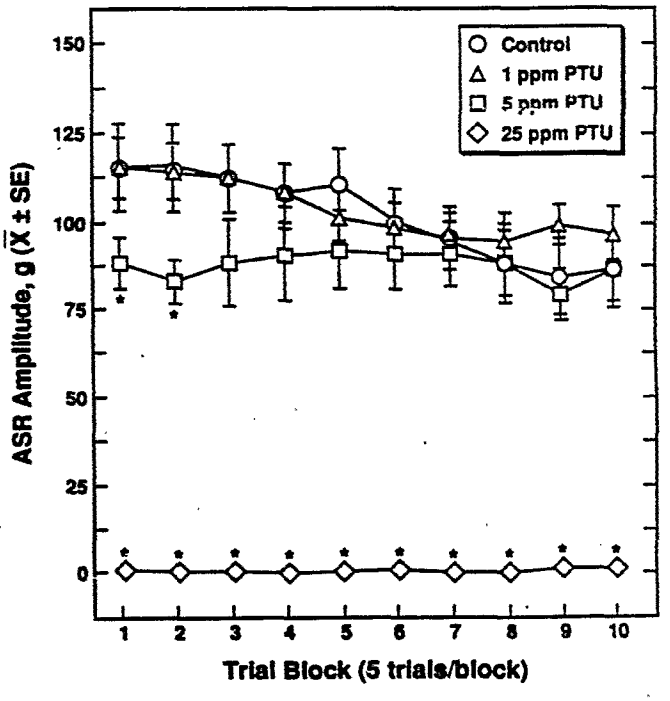

FIG. 6. Acoustic startle habituation testing on PND 24 revealed that animals exposed to the highest dose of PTU did not show a startle response, and that animals from the intermediate dose of PTU showed reduced amplitudes in early trials and did not appear to habituate to the stimulus across trial blocks (five trials/block). $N = 6$, 7, 8, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively (one male and one female/litter). *Significantly different from the control value during a particular trial block ($p < 0.05$).

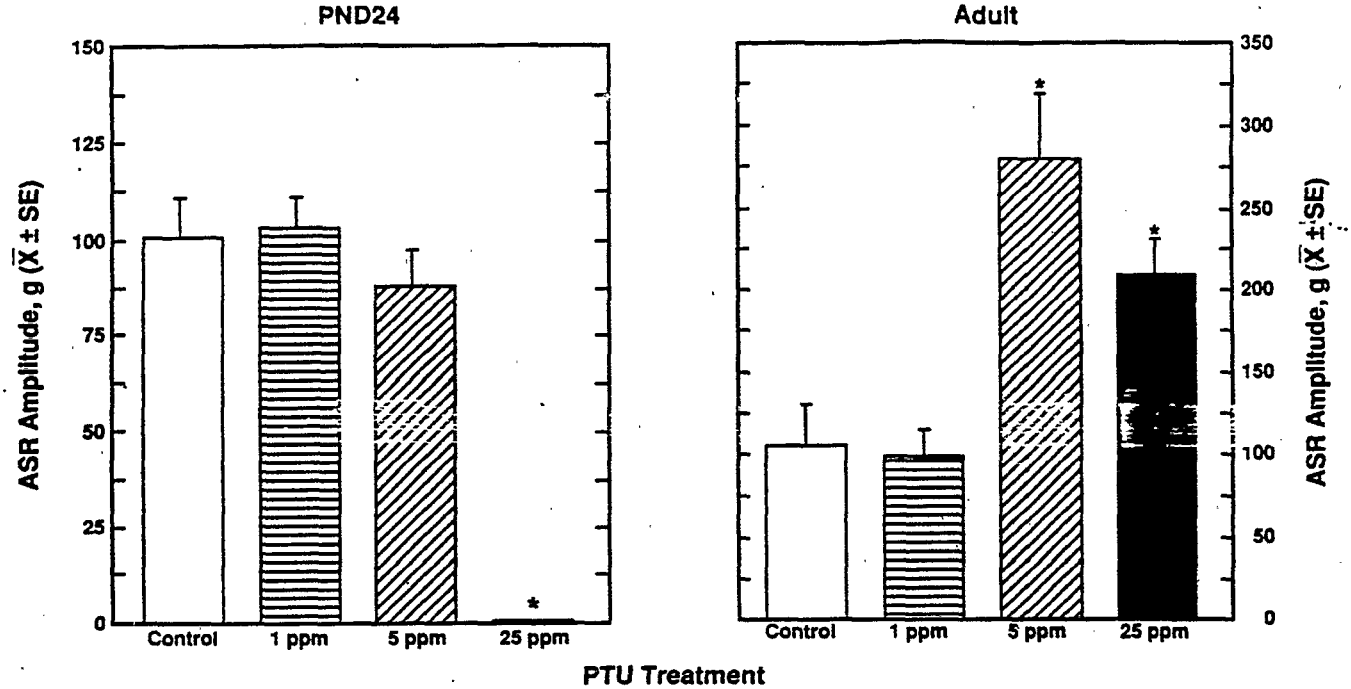

FIG. 7. Acoustic startle response amplitudes (mean ± SE), zero in pups from the 25 ppm dose on PND 24 (left), were significantly higher in adult 5 or 25 ppm PTU offspring compared to adult controls (right). *Significantly different from the age-matched control value [N = 6, 7, 8, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively (one male and one female/litter)] ($p < 0.05$).

motor activity. However, functional auditory impairment was evident in adult offspring from both the intermediate and the higher dose of PTU, indicating that the developing auditory system may be particularly sensitive to, and permanently affected by, perinatal hypothyroidism.

The effects of PTU on motor activity were age and dosage dependent. Assessments of young offspring (≤PND 21) treated with ≥5 ppm PTU showed transiently reduced activity compared to the controls, a finding which suggests a PTU-induced delay in the ontogenetic profile of motor activity in these offspring. The overt balance and coordination deficits observed in our animals at 25 ppm PTU may also have influenced motor activity performance in the figure-eight maze. These coordination and/or activity problems may be related to the effects of developmental hypothyroidism on neurotransmitter systems (Rastogi *et al.*, 1976; Dupont *et al.*, 1981), on cerebellar neuron arborization, myelination, and synaptogenesis (Poddar and Kumar Sarkar, 1993; Rabie *et al.*, 1979; Vincent *et al.*, 1982), and development of the peripheral vestibular system (Demêmes *et al.*, 1986).

In contrast to the decrease in activity seen in young hypothyroid animals, older animals (≥PND 30) from the 25 ppm PTU group demonstrated a distinct, and persistent, increase in motor activity. In a study similar to our own, Long–Evans rats, exposed to PTU from birth to PND 25, were hyperactive when tested at 50 and at 100 days of age (Tamasy *et al.*, 1986). However, whereas these authors reported that the hyperactivity appeared to lessen in magnitude with age, our animals were increasingly hyperactive with advancing age, a difference which may be attributable to the particular tests which were employed. It would have been of interest to follow thyroid hormone concentrations following cessation of PTU exposure in our study to determine if some effects seen at older ages might be exacerbated by incomplete recovery, and/or overcompensation, of thyroid function. Tamasy *et al.* (1986) showed that T4 concentrations remained significantly reduced on PND 50, whereas they were not different from controls on PND 100. Results from the current study, and previous reports (Davenport and Hennies, 1976; Schalock *et al.*, 1977; Tamasy *et al.*, 1986), underscore the importance of evaluating motor activity at numerous developmental ages as activity patterns resulting from hypothyroidism appear to be age dependent.

Whereas the acoustic startle response appears by PND 13 in normal animals (Sheets *et al.*, 1988), we found a profound delay (>11 days) in the development of the startle response in pups from the 25 ppm PTU dosage group. It is likely that PTU delayed the development of sensory and/or motor processes necessary for responding to an acoustic startle stimulus. Related work has shown that pups made hypothyroid by methimazole (0.1% in dam's drinking water until PND 10) showed a 6-day delay in startle development (Schneider and Golden, 1986), whereas thiourea exposure (po to dams from GD 18 to PND 10) caused a 3-day delay

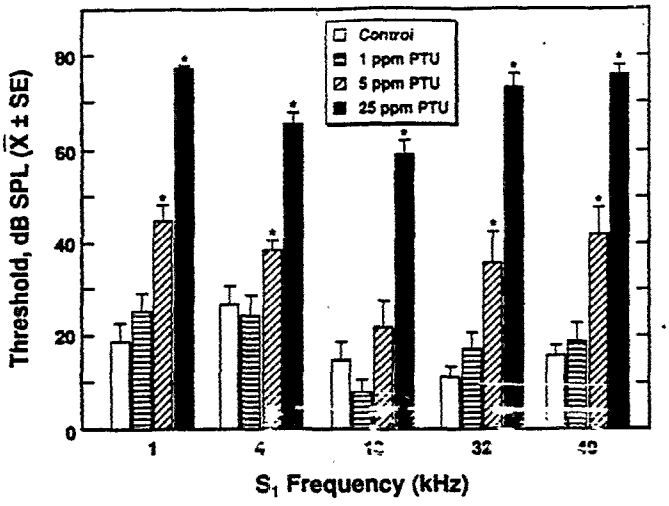

FIG. 8. Auditory thresholds were significantly elevated by ≥5 ppm PTU. Severe hearing deficits were detected at all frequencies for the highest dose of PTU (25 ppm). At 5 ppm PTU, auditory thresholds were significantly elevated compared to controls for all but the 16 kHz frequency. $N = 6, 7, 8$, and 8 litters per treatment for control, 1, 5, and 25 ppm, respectively (one male and one female/litter). For each frequency, asterisks indicate that the value was significantly different from the control value ($p < 0.05$).

in the development of the startle response (Schneider and Golden, 1987). Methimazole, thiourea, and PTU all inhibit the formation of thyroid hormones, but PTU is unique in that it also inhibits the peripheral deiodination of T4 to T3 (Geffner *et al.*, 1975). Therefore, the aggressive mechanism of action of PTU, and the longer exposure duration used in the present study, may be responsible for the more prolonged delay in startle development seen in our study compared to those in other studies. Delayed development of the startle response may be a particularly sensitive indicator of perinatal hypothyroidism, and significant delays in its development may have been missed in the lower dosage groups because the endpoint was only evaluated on PND 24. Therefore, testing animals at a number of developmental ages from PND 13 through adulthood would provide valuable information on the relative sensitivity of the ontogeny of the startle response to lowered levels of thyroid hormone(s).

When tested as adults, offspring from the 5 and 25 ppm treatment groups showed significantly elevated startle response amplitudes compared to the controls. This finding is particularly interesting as it suggests that hyperreactivity occurs following the developmental delay in the response. In normal rats, a number of regions important in the acoustic startle pathway (Davis *et al.*, 1982) are undergoing rapid development during the postnatal period. Therefore, neurohistological and functional (i.e., acoustic startle response) comparisons of normal, hypothyroid; and thyroxin-"replaced" offspring (e.g., Uziel *et al.*, 1985) at a number of ages should be useful in mapping the development of this pathway and in identifying which regions may be particularly dependent on thyroid hormones for normal development.

Reflex modification procedures demonstrated overt, 50- to 60-dB, auditory threshold deficits at all frequencies tested for animals from the 25 ppm PTU group as well as 20- to 25-dB deficits at the 5 ppm PTU treatment. The animals in this latter treatment group did not demonstrate the overt symptoms of hypothyroidism seen at the higher dose; indeed, the hearing deficits and elevated acoustic startle amplitudes detected in adult animals were the only prominent functional effects detected for this treatment group. Such findings indicate that the developing auditory system, and/or associated pathways, may be particularly vulnerable to the effects of hypothyroidism, and that the effects on these areas are persistent. Studies of laboratory rodents have demonstrated that severe hypothyroidism causes morphological abnormalities in the organ of Corti (Deol, 1973; Uziel *et al.*, 1981, 1983, 1985), as well as functional auditory deficits (Uziel *et al.*, 1980, 1985). In the absence of thyroid hormone supplementation during critical periods of cochlear development, the effects of hypothyroidism on many cochlear structures are irreversible (Uziel *et al.*, 1985; Uziel, 1986). Comparative anatomical investigations of auditory structures between normal animals and animals suffering moderate to severe reductions in thyroid hormones would provide information on which auditory structures may be particularly sensitive to lowered thyroid hormone levels.

Our findings suggest that, at moderate dosages of PTU, compensatory mechanisms may be triggered which attenuate many of the effects of lowered thyroid hormone levels. Despite the sharp reductions in circulating T4 concentrations in the 5 ppm PTU group, the T3 concentrations were relatively unaffected, a finding which may explain the general absence of effects on physical development in the 5 ppm group compared to the 25 ppm group This finding underscores the importance of monitoring both hormones, as it is sometimes assumed that as T4 concentrations decline, T3 levels are similarly reduced (Blake and Henning, 1985). One such compensatory mechanism may be related to the intracellular metabolism of T4. The enzyme which converts T4 to T3 in the brain, type II monodeiodinase (5′-D-II), differs from the enzyme which performs this function in the periphery, type I monodeiodinase (5′-D-I). The 5′-D-II, which appears to be unique to the brain, anterior pituitary, brown fat, and placenta (Edmonds, 1987), undergoes a compensatory increase in response to lowered thyroxin levels (Silva and Larsen, 1982), and thus may protect the brain from moderate reductions in circulating T4 levels by maintaining cellular T3 levels near normal (Edmonds, 1987). While it is not currently known which enzyme is functioning in peripheral auditory structures, if dependent on 5′-D-I, peripheral structures may be less able to compensate for reduced T4 because, unlike 5′-D-II, this enzyme is not induced when T4 levels fall. Furthermore, PTU has been shown to block the conver-

sion of T4 to T3 by directly inhibiting 5′-D-I (Braverman and Ingbar, 1962; Oppenheimer *et al.*, 1972; Geffner *et al.*, 1975), whereas PTU has no direct effect on 5′-D-II. Thus, in the case of PTU, hypothyroidism-induced damage to peripheral auditory structures may be further exacerbated by PTU's direct inhibition of 5′-D-I. These factors may aid in explaining why the effects of PTU on auditory function may be particularly acute relative to other agents which cause a similar pattern of hormone reduction, such as polychlorinated biphenyls (see Goldey *et al.*, 1995).

We have demonstrated that measures of auditory function, motor activity, and growth are differentially affected by reductions in circulating thyroid hormone concentrations, and our findings suggest that auditory processes may be particularly sensitive to the effects of hypothyroidism. In most developed countries, newborns are typically screened for congenital hypothyroidism (LaFranchi, 1993), and follow-up audiometric assessments have found sensorineural hearing loss in infants and children with severe congenital hypothyroidism (e.g., endemic cretins; Debruyne *et al.*, 1983) or Pendred's syndrome (Fraser, 1965; Batsakis and Naishiyana, 1962; Coakley *et al.*, 1992). However, if developing auditory structures are sensitive to nominal, and clinically undetected, changes in thyroid status (as may result from moderate dietary iodine deficiency during prenatal and postnatal development, early stages of myxedema, and/or exposure to thyrotoxic environmental agents), resultant hearing problems may be more subtle and, thus, may be overlooked. Undetected hearing loss in children may cause impaired speech development, attention deficits, and educational delays; therefore, early detection and intervention for even mild thyroid dysfunction may be crucial for normal childhood development.

ACKNOWLEDGMENTS

The authors thank Dave Ellis and Joy Hein for their excellent technical assistance on this project. We also thank Drs. Chris Lau, Eias Zahalka, and Stan Barone, Jr. for their advice and support and Drs. Chris Lau, Lawrence Fechter, and Alain Uziel for reviewing an earlier version of the manuscript.

REFERENCES

Avery, D. L., and Spyker, J. M. (1977). Foot tattoo of neonatal mice. *Lab. Anim. Sci.* **27,** 110–112.

Balázs, R., Kovács, S., Teichgräber, P., Cocks, W. A., and Eayrs, J. T. (1968). Biochemical effects of thyroid deficiency on the developing brain. *J. Neurochem.* **15,** 1335–1349.

Batsakis, J. G., and Nishiyama, R. H. (1962). Deafness with sporadic goiter (Pendred's syndrome). *Arch. Otolaryngol.* **76,** 401–406.

Behnam-Rassoli, M., Herbert, L. C., Howard, V., Pharoah, P. O. D., and Stanisstreet, M. (1991). Effect of propylthiouracil treatment during prenatal and early postnatal development on the neocortex of rat pups. *Neuroendocrinology* **53,** 321–327.

Blake, H. H., and Henning, S. J. (1985). Effect of propylthiouracil dose on serum thyroxine, growth and weaning in young rats. *Am. J. Physiol.* **248,** R524–R530.

Boyages, S. C. (1993). Clinical review 49: Iodine deficiency disorders. *J. Clin. Endocrinol. Metab.* **77,** 587–591.

Boyages, S. C., and Halpern, J. P. (1993). Endemic cretinism: Toward a unifying hypothesis. *Thyroid* **3,** 59–69.

Braverman, L. E., and Ingbar, S. H. (1962). Effects of propylthiouracil and thiouracil on the metabolism of thyroxine and several of its derivatives by rat kidney slices in vitro. *Endocrinology* **71,** 701–712.

Coakley, J. C., Keir, E. H., and Connelly, J. F. (1992). The association of thyroid dyshormonogenesis and deafness (Pendred syndrome): Experience of the Victorian Neonatal Thyroid Screening Programme. *J. Paediatr. Child Health* **28,** 398–401.

Comer, C. P., and Norton, S. (1982). Effects of perinatal methimazole exposure on a developmental test battery for neurobehavioral toxicity in rats. *Toxicol. Appl. Pharmacol.* **63,** 133–141.

Cooke, P. S., Kirby, J. D., and Porcelli, J. (1993). Increased testis growth and sperm production in adult rats following transient neonatal goitrogen treatment: Optimization of the propylthiouracil dose and effects of methimazole. *J. Reprod. Fertil.* **97,** 493–499.

Crofton, K. M. (1992). Reflex modification and the assessment of sensory dysfunction. In *Neurotoxicology* (H. Tilson and C. Mitchell, Eds.), pp. 181–211. Raven Press, New York.

Crofton, K. M., Dean, K. F., Menache, M. G., and Janssen, R. (1990). Trimethyltin effects on auditory function and cochlear morphology. *Toxicol. Appl. Pharmacol.* **105,** 123–132.

Crofton, K. M., and Zhao, X. (1993). Mid-frequency hearing loss in rats following inhalation exposure to trichloroethylene: Evidence from reflex modification audiometry. *Neurotoxicol. Teratol.* **15,** 413–423.

Davenport, J. W., and Hennies, R. S. (1976). Perinatal hypothyroidism in rats: Persistent motivational and metabolic effects. *Dev. Psychobiol.* **9,** 67–82.

Davis, M., Gendelman, D. S., Tischler, M. D., and Gendelman, P. M. (1982). A primary acoustic startle circuit: Lesion and stimulation studies. *J. Neurosci.* **2,** 791–805.

Debruyne, F., Vanderschueren-Lodeweyckx, M., and Bastijns, P. (1983). Hearing in congenital hypothyroidism. *Audiology* **22,** 404–409.

Delong, G. R. (1993). Effects of nutrition on brain development in humans. *Am. J. Clin. Nutr.* **57,** 286S–290S.

Demêmes, D., Desmesne, C., Legrand, C., and Sans, A. (1986). Effects of hypothyroidism on postnatal development in the peripheral vestibular system. *Dev. Brain Res.* **25,** 147–152.

Deol, M. S. (1973). An experimental approach to the understanding and treatment of hereditary syndromes with congenital deafness and hypothyroidism. *J. Med. Genet.* **10,** 235–242.

Dupont, A., Dussault, J. H., Rouleau, D., DiPaolo, T., and Coulombe, P. (1981). Effect of neonatal thyroid deficiency on the catecholamine, substance P, and thyrotropin-releasing hormone contents of discrete rat brain nuclei. *Endocrinology* **108,** 2039–2045.

Dussault, J. H., and Ruel, J. (1987). Thyroid hormones and brain development. *Annu. Rev. Physiol.* **49,** 321–334.

Edmonds, C. J. (1987). Peripheral metabolism of thyroxine. *J. Endocrinol.* **114,** 337–339.

Engler, H., Taurog, A., and Dorris, M. L. (1982). Preferential inhibition of thyroxine and 3,5,3′-triiodothyronine formation by propylthiouracil and methylmercaptoimidazole in thyroid peroxidase-catalyzed iodination of thyroglobulin. *Endocrinology* **110,** 190–197.

Fraser, G. R. (1965). Association of congenital deafness with goiter (Pendred's syndrome). *Ann. Hum. Genet.* **28,** 201–249.

Geffner, D. L., Azukizawa, M., and Hershman, J. M. (1975). Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin secretion in man. *J. Clin. Invest.* **55,** 224–229.

Goldey, E. S., Kehn, L. S., Lau, C., Rehnberg, G. L., and Crofton, K. M.

(1995). Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. *Toxicol. Appl. Pharmacol.* **135,** 77–88.

Halpern, R., Cooper, D. S., Kieffer, J. D., Saxe, V., Mover, H., Maloof, F., and Ridgway, E. C. (1983). Propylthiouracil (PTU) pharmacology in the rat. I. Serum and thyroid PTU measurements by radioimmunoassay. *Endocrinology* **113,** 915–920.

LaFranchi, S. (1993). American Academy of Pediatrics, American Thyroid Association: Newborn screening for congenital hypothyroidism: Recommended guidelines. *Thyroid* **3,** 257–263.

Narayanan, C. H., Narayanan, Y., and Browne, R. C. (1982). Effects of induced thyroid deficiency on the development of suckling behavior in rats. *Physiol. Behav.* **29,** 361–370.

Oppenheimer, J. H., Schwartz, H. L., and Surks, M. I. (1972). Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. An explanation for the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. *J. Clin. Invest.* **51,** 2493–2497.

Poddar, R., and Sarkar, P. K. (1993). Delayed detyrosination of alpha-tubulin from parallel fibre axons and its correlation with impaired synaptogenesis in hypothyroid rat cerebellum. *Brain Res.* **614,** 233–240.

Rabie, A., Favre, C., Clavel, M. C., and Legrand, J. (1979). Sequential effects of thyroxine on the developing cerebellum of rats made hypothyroid by propylthiouracil. *Brain Res.* **161,** 469–479.

Rastogi, R. B., Lapierre, Y., and Singhal, R. L. (1976). Evidence for the role of brain biogenic amines in depressed motor activity seen in chemically thyroidectomized rats. *J. Neurochem.* **26,** 443–449.

Rodriguez-Péna, A., Ibarrola, N., Iñiguez, M. A., and Bernal, J. (1993). Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. *J. Clin. Invest.* **91,** 812–818.

Ruppert, P. H., Dean, K. F., and Reiter, L. W. (1984a). Development of locomotor activity of rat pups in figure-eight mazes. *Dev. Psychobiol.* **18,** 247–260.

Ruppert, P. H., Dean, K. F., and Reiter, L. W. (1984b). Trimethyltin disrupts acoustic startle responding in adult rats. *Toxicol. Lett.* **22,** 33–38.

SAS (1989). *SAS/STAT User's Guide,* Vol. 2, Version 6, 4th ed., SAS Institute, Cary, NC.

Schalock, R. L., Brown, W. J., and Smith, R. L. (1977). Neonatal hypothyroidism: Behavioral, thyroid hormonal and neuroanatomical effects. *Physiol. Behav.* **19,** 489–491.

Schneider, B. F., and Golden, W. L. (1986). Acquisition of acoustic startle shows a dose–response to serum free T4. *Int. J. Dev. Neurosci.* **4,** 397–400.

Schneider, B. F., and Golden, W. L. (1987). Acquisition of acoustic startle response in relation to growth and thyroid function in rats. *Int. J. Dev. Neurosci.* **5,** 99–106.

Sheets, L. P., Dean, K. F., and Reiter, L. W. (1988). Ontogeny of the acoustic startle response and sensitization to background noise in the rat. *Behav. Neurosci.* **102,** 706–713.

Silva, J. E., and Larsen, P. R. (1982). Comparison of iodothyronine 5′-deiodinase and other thyroid-hormone-dependent enzyme activities in the cerebral cortex of hypothyroid neonatal rat: Evidence for adaptation to hypothyroidism. *J. Clin. Invest.* **70,** 1110–1115.

Sorcini, M., Balestrazzi, P., Grandolfo, M. E., Carta, S., and Giovannelli, G. (1993). The National Register of infants with congenital hypothyroidism detected by neonatal screening in Italy. *J. Endocrinol. Invest.* **16,** 573–577.

Tamasy, V., Meisami, E., Vallerga, A., and Timiras, P. S. (1986). Rehabilitation from neonatal hypothyroidism: Spontaneous motor activity, exploratory behavior, avoidance learning and responses of pituitary–thyroid axis to stress in male rats. *Psychoneuroendocrinology* **11,** 91–103.

Uziel, A., Rabie, A., and Marot, M. (1980). The effect of hypothyroidism on the onset of cochlear potentials in developing rats. *Brain Res.* **182,** 172–175.

Uziel, A., Gabrion, J., Ohresser, M., and Legrand, C. (1981). Effects of hypothyroidism on the structural development of the organ of Corti in the rat. *Acta Otolaryngol.* **92,** 469–480.

Uziel, A., Legrand, C., Ohresser, M., and Marot, M. (1983). Maturational and degenerative processes in the organ of Corti after neonatal hypothyroidism. *Hearing Res.* **11,** 203–218.

Uziel, A., Legrand, C., and Rabie, A. (1985). Corrective effects of thyroxine on cochlear abnormalities induced by congenital hypothyroidism in the rat. I. Morphological study. *Dev. Brain Res.* **19,** 111–122.

Uziel, A. (1986). Periods of sensitivity to thyroid hormone during the development of the organ of Corti. *Acta Otolaryngol. (Stockh). Suppl.* **429,** 23–27.

Vaccari, A., Bonsignori, Z. L., deMontis, G., Stefanini, E., Martino, E., and Gessa, G. L. (1990). Neonatal hypothyroidism induces striatal dopaminergic dysfunction. *Neuroscience* **35,** 699–706.

Vanderschueren-Lodeweyckx, M., Debruyne, F., Dooms, L., Eggermont, E., and Eeckels, R. (1983). Sensorineural hearing loss in sporadic congenital hypothyroidism. *Arch. Dis. Child* **58,** 419–422.

Vincent, J., Legrand, C., Rabie, A., and Legrand, J. (1982). Effects of thyroid hormone on synaptogenesis in the molecular layer of the developing rat cerebellum. *J. Physiol. (Paris)* **78,** 729–738.

Young, L. S., and Fechter, L. D. (1983). Reflex inhibition procedures for animal audiometry: A technique for assessing ototoxicity. *J. Acoust. Soc. Am.* **73,** 1686–1693.